Silencing of SCD and SREBP1 Genes by Short Hairpin RNAs in Chicken Hepatic Cells in Vitro

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Abstract

RNA interference by short hairpin RNAs (shRNAs) is a widely used post transcriptional silencing mechanism for suppressing expression of the target gene. In the current study, five shRNA molecules each against *SCD* and *SREBP1* genes involved in *denovo* lipid biosynthesis were designed upon considering parameters such as secondary structures of shRNAs, mRNA target regions, GC content and thermodynamic properties (ΔG overall, ΔG duplex and ΔG break-target), synthesized and cloned in pENTR/U6 entry vector to knockdown the expression of *SCD* and *SREBP1* genes. After transfection of these shRNA constructs into the chicken embryonic hepatocytes, expressions of the target genes were monitored by real time PCR. Significant reduction ($P < 0.05$) in the expression of *SCD* and *SREBP1* genes was observed in hepatocytes. The shRNAs against *SCD* gene showed the knock down efficiency ranged from 20.4% (shRNA5) to 74.2% (shRNA2). In case of *SREBP1* gene, the shRNAs showed knock-down efficiency ranging from 26.8% (shRNA4) to 95.85% (shRNA1). The shRNAs against both the genes introduced in chicken hepatocyte cells did not show any significant impact on expression of immune response genes (IFNA and IFNB) in those cells. These results clearly demonstrated the successful down regulation of the expression of *SCD* and *SREBP1* genes by the shRNA molecules against both the target genes under *in vitro* condition. It is concluded that the shRNA molecules against *SCD* and *SREBP1* genes showed great potential to silence the expression of these genes under *in vitro* chicken embryonic hepatocyte cells.

Introduction

Genetic selection has greatly improved chicken production over the last 50 years which provides high quality dietary protein to mitigate our protein demand. However, intensive genetic selection for rapid growth inadvertently selected for increased carcass fat. Carcass fat in broilers at 6 weeks of age accounts for 10–15% of the total carcass weight and deposits at a rate of up to 6 g fat/kg body wt/day between 42 and 49 days of age [1]. The predominant site of adipose deposition in chickens is abdominal fat and, to a less extent, subcutaneous fat and intramuscular fat [2]. This is probably because abdominal fat is proportionately the largest when body weights and growth rates are the critical traits under selection. Excess fat is an economic loss to the broiler industry by reducing the conversion of feed to meat, and the disposal of fat leads to additional economic loss. Excess fat may also contribute to the development of other detrimental traits, such as reduced reproductivity and immune competence, which consequently affect growth and yield of meat [3].

Fatty acids in animals can come from diet or be synthesized from glucose in lipogenic tissues such as liver and adipose tissues catalyzed by fatty acid synthase. Dietary fatty acids are absorbed by enterocytes, packaged with cholesterol, lipoproteins and other lipids into chylomicrons, and transported into circulation. Lipids synthesized in the liver are packaged into LDLs and released into the blood directly. TAG-rich VLDL are taken up by adipocytes and deposited into adipose tissue. In chickens, the contribution of portomicrons (similar to chylomicrons in mammals) from dietary fats to triglyceride is low, since there is only 5% of dietary lipids in regular feed [4]. Triglyceride (TAG) deposits in chicken adipose tissue mainly through the uptake of fatty acids from circulating TAG-rich VLDL synthesized by the liver. Increased VLDL levels were found virtually in chickens associated with high fatness, reflecting the critical role of the liver in fatness of chickens. The contribution of *de novo* lipogenesis in liver to fat deposition was also confirmed in chicken [5]. Only 15% of *de novo* lipogenesis occur in adipose tissue, and the rest is happened in liver [6]. The product of *de novo* lipogenesis in liver are secreted in the form of VLDL and delivered to other tissues. However, the basic step in the synthesis of lipids is the formation of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), the major fatty acid species in mammalian cell lipids.

One key regulator of the fatty acid composition of cellular lipids are Stearoyl-CoA desaturases (*SCD*), the endoplasmic reticulum-resident enzyme that catalyze the introduction of the first double bond in the *cis*- Δ^9 position of several saturated fatty acyl-CoAs, principally palmitoyl-CoA and stearoyl-CoA, to yield palmitoleoyl- and oleoyl-CoA, respectively [7, 8]. Stearoyl-CoA desaturase (*SCD*) is an iron-containing enzyme that catalyzes a rate-limiting step in synthesis of the unsaturated fatty acids. The principal product of *SCD* is oleic acid, which is formed by desaturation of stearic acid.

Sterol regulatory element binding protein-1 (*SREBP1*) is a transcription factor that binds to a sequence in the promoter of a group of genes, called sterol regulatory element-1 (SRE1). This element is a decamer flanking the LDL receptor gene and other genes involved in sterol biosynthesis. The *SREBP1* regulates genes required for glucose metabolism and fatty acid and lipid production and its expression is regulated by insulin [9]. The *SREBP1* regulates genes related to lipid and cholesterol production and its activity is regulated by sterol levels in the cell [10, 11]. The transcription factor *SREBP1* regulates *de novo* lipogenesis in the liver in response to increases in insulin. SREBPs are transcription factors of the basic helix-loop-helix leucine zipper family that are synthesized as precursors and bound to the endoplasmic reticulum membrane. In the present study, we have developed an *in vitro* model for reduction of *SCD* and *SREBP1* expression in chicken embryonic hepatic cells by the short hairpin RNA molecules.

Materials And Methods

Experimental animals

The study was conducted in the control broiler chicken line maintained at ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, India. The fertile eggs were collected from control broiler chicken and incubated at 98°F and 85% relative humidity in the incubator for 12 to 13 days. The embryonated eggs of 12-13 days old were taken out for preparation of embryonic hepatocyte primary cell culture. The whole study was approved by the Institute Animal Ethics Committee (IAEC) and Institute Bio-safety Committee (IBSC) of ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, India to carry out the animal experiment.

Designing of shRNA molecules:

A total of 5 shRNA molecules each were designed from ORF of *SCD* (NCBI Accession No. NC_006093) and *SREBP1* (NCBI Accession No. NC_006101) genes using BLOCK-IT RNAi Designer (<https://rnaidesigner.thermofisher.com/rnaexpress/>) software (Table 1). To determine efficiency of shRNA molecules, secondary structures of shRNAs were predicted by *in silico* analysis in the web server of Mfold 2.3 version [12] (<http://mfold.mna.albany.edu/?q=mfold/RNA->

Folding-Form) was used after considering the all the required criteria. Secondary structures of antisense strands of shRNA molecules against *SCD* and *SREBP1* genes were predicted by using the RNA fold program of the Vienna RNA web service version 2.0 [13] (<http://ma.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>). Further, Thermodynamic properties that governs the binding affinity of shRNA molecules and its target region on mRNA molecules [15] were predicted by Oligowalk program [14] in the RNA structure version 5.3 (http://ma.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi). Following thermodynamic properties [16] were included in the present study:

- i. **ΔG overall**: The net energy (ΔG in kcal/mol) resulting due to binding of target site by oligos, after consideration of all energy contributions viz. oligo-self-structure energy and target structure breaking energy. The higher negative value of ΔG signifies stronger binding.
- ii. **ΔG duplex**: It determines the stability of the duplex formed between guide strand of siRNA and the nucleotides at the target site. A less negative value of ΔG duplex denotes less stability of duplex and vice versa.
- iii. **ΔG break-target (disruption energy)**: The energy cost for disrupting local secondary structure at the mRNA target region. A more negative value denotes that the binding site is not completely open and less accessible.

Cloning of shRNAs:

A final concentration of 50 μM double stranded oligos were prepared by mixing top strand DNA oligo (200 μM) – 5 μl , Bottom strand DNA oligo (200 μM) - 5 μl , 10X Oligo annealing buffer - 2 μl and DNase/RNase free water - 8 μl , by using the conditions 95°C for 4 minutes and then allowed to cool at room temperature for 5-10 minutes to obtain double-stranded oligos to anneal.

The ds oligos (50 μM stock) were diluted to a final concentration of 5 nM by performing two 100-fold serial dilutions. First 100-fold dilution was accomplished with DNase/RNase-free water and then second 100-fold dilution was completed with 1X Oligo annealing buffer supplied with the kit. These diluted ds oligos were used for cloning into the pENTR /U6 Entry Vector (Figure.S1). The annealed shRNA oligo nucleotides were ligated into RNAi Ready pENTR™ U6 vector by preparing 20 μl of ligation mixture containing 5X Ligation Buffer - 4 μl , pENTRTM /U6 (0.5ng/ μl) - 2 μl , ds oligo (5 nM; 1:10000 dilution) - 1 μl , T4 DNA Ligase (1 U/ μl) - 1 μl and DNase/RNase free water - 12 μl . This ligation mixture was mixed well and incubated for 30 minutes at room temperature. Scrambled shRNA oligos (lacZ) supplied with the kit were also ligated to pENTR™/U6 vector and used as a negative control in the experiment.

Recombinant vector containing the shRNA molecules were transformed into the One Shot® TOP10 chemically competent *E.coli* cells by giving heat-shock (42°C for 30 seconds and then, immediately transferred to ice). Transformed cells were grown in 250 μl of super optimal broth with catabolite repression (S.O.C.) medium at room temperature by incubating in horizontal shaker incubator (200 RPM) at 37°C for 1 hour. 200 μl of transformation mixture was spread on a pre-warmed LB agar plate containing 50 $\mu g/ml$ of kanamycin and incubated overnight at 37°C.

Kanamycin resistant colonies were picked and inoculated into the LB broth containing kanamycin 50 $\mu g/ml$ of media and incubated over night at 37°C to analyze the positive clones. Kanamycin resistant clones were screened for the presence of shRNA in the plasmid constructs by performing colony PCR with U6 forward primer: 5'GGACTATCATA TGCTTACCG3' and M13 reverse primer: 5'-CAGGAAACAGCTATGAC-3' by using 2.5 μl of 10XPCR buffer, 0.5 μl of dNTP mix (2.5 mM), 1.5 μl (30 ng) each of forward and reverse primers, 0.2 μl (1U) of Taq DNA polymerase, 2 μl of colony lysate and nuclease free water to make the volume up to 25 μl in 0.2 ml PCR tubes. Thermal cycling conditions followed were, initial denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 30seconds, primer annealing of 54°C for 30 seconds and extension at 72°C for 30 seconds and final extension of 72°C for 10 minutes.

Plasmids containing shRNA molecules against *SCD* and *SREBP1* genes were isolated by using Gene JET Plasmid Miniprep Kit (#K0503, Thermo Scientific, USA). The plasmid obtained from each pENTR™/U6 entry construct was sequenced to confirm the sequence and correct orientation of the ds oligo insert.

Establishment of primary chicken embryonic hepatocyte (CEH) culture:

Primary chicken embryonic hepatocyte culture was established by using 12-13 days old embryos. Embryos were collected aseptically by breaking the broad end of the egg after piercing the chorio-allantoic membrane (CAM) and placed in 9 cm petri-dish containing sterile Phosphate Buffer Saline (PBS) and rinsed thoroughly. Head, limbs and wings were separated and then, ventral side of the embryo was cut opened for collecting the liver lobes into the petri-dish containing the PBS. After thorough washing in PBS and mincing, liver tissues were transferred into the beaker containing sterile magnetic bar and approximately 10-15 ml of 0.125% trypsin. The beaker was placed on the magnetic stirrer for stirring at 37°C at about 100 RPM for less than 10 minutes. After allowing the pellets to settle down, supernatant was filtered through sterile double layered muslin cloth into a fresh beaker. The filtrate was centrifuged for 5 minutes at 3000 RPM and to stop the trypsin action, the resulting pellet was re-suspended in 5ml of growth medium (DMEM, Sigma) containing Fetal Bovine Serum (FBS). Now, the media with the liver cells were centrifuged at 3000 RPM for 3 min and then, the resulting pellet was re-suspended in 5ml of growth medium containing 10% FBS, 1% Tryptone Phosphate Broth and antibiotic-antimycotic solution. Haemocytometer was used to count the number of cells and accordingly, the cell suspension was diluted to get a cell concentration of 1×10^6 cells/ml. Approximately 2×10^5 cells/ cm^2 were seeded into the 25 cm^2 tissue culture flask and incubated at 37°C with 5% CO₂. Medium was changed at regular intervals to have good growth of cells and counter the depression of pH.

Transfection of shRNA constructs into CEH cells:

All the shRNA recombinant plasmid constructs against *SCD* and *SREBP1* genes were transfected into the chicken primary embryonic hepatocytes in order to assess the activity of shRNA molecules. Approximately 0.4 ml of the hepatocyte cell suspension and plasmid containing shRNA molecules against *SCD* and *SREBP1* genes were taken into electroporation cuvette and mixed gently. Single square wave pulse was given at a voltage of 150 mV for pulse length of 10 milli second. Both, pENTR U6/lacZ shRNA and pcDNA 1.2/V5/lacZ reported plasmids were co-transfected in to CEH. The pcDNA 1.2/V5/lacZ reported plasmid is used as a positive control for RNAi response in CEH. For getting optimal results, we had used 6 fold more entry construct DNA than reporter plasmid during

co-transfection. Immediately after transfection, approximately 200 µl of cell suspension transferred to each well of a well plate containing 1.8 ml of growth medium and incubated at 37°C with 5% CO₂ level.

RNA extraction and cDNA synthesis;

The cells were harvested after 48 hours of transfection and RNA was isolated for transient RNAi analysis. The total RNA was isolated from the hepatic cells grown after successful transfection of the plasmids containing shRNA molecules against both target genes as per the manufacturer's protocol using Trizol (Sigma). The RNA samples were treated with DNase I (Fermentas) for removal of possible genomic DNA contamination. cDNA was synthesized by using High-Capacity cDNA Reverse transcription Kit (Applied Biosystems, #4368814) in a final volume of 20 µL containing 10X Reverse Transcription (RT) buffer (2 µL), 10X RT random primers (2 µL), 100 mM of 25X dNTP mix (0.8 µL), RNase inhibitor (1 µL), MultiScribe™ Reverse Transcriptase (1 µL), Nuclease-free H₂O (3.2 µL) and 1 ng RNA (10 µL). Reverse transcription was carried out in thermocycler (Mastercycler, Eppendorf, Germany) following the manufacturer's instructions, which includes the incubation of reaction at 25°C for 10 minutes followed by 37°C for 2 hours and 85°C for 5 minutes. The resulted cDNA was stored at -20°C till further use.

Real time quantitative PCR:

The mRNA expression levels of *SCD*, *SREBP1* genes and immune response genes viz. Interferon-A (IFN-A) and Interferon-B (IFN-B) genes in the transfected hepatic cells were quantified by using thermal cycler Applied Biosystems® Step One Real-Time PCR (Life Technologies) with SYBR® Green JumpStart™ TaqReadyMix™. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for normalizing different amounts of input RNA (Table.2). The realtime PCR was performed for each sample in duplicates. Amplification curves for both the target genes showed typical initiation phase, exponential and plateau phase, indicating the successful exponential amplification of the product (Figure.S2). Melt curves of the amplified products displayed specific single peak for *SCD*, *SREBP1* and *GAPDH* genes, indicating specific amplification and homogeneity of the PCR products. These products were run on 1.5% agarose gel and expected amplicons sizes were observed.

The gene quantification was expressed as “n-fold up/down regulation of transcription” in relation to a reference sample, called the calibrator (mock transfected hepatocytes). The expression of target gene was calibrated by that of the reference gene, *GAPDH*, at each time point and converted to the relative expression (fold of expression), as follows:

$$\text{Fold of expression} = 2^{-\Delta\Delta Ct}$$

Where,

ΔCt = Average Ct of target gene - Average Ct of reference gene (*GAPDH*)

$\Delta\Delta Ct$ = Average ΔCt of target (shRNA) – Average ΔCt of calibrator sample (Mock)

The Knock down efficiency (KD%) of all the shRNA was calculated by considering scrambled shRNA as control.

Western Blotting for detection of SCD and SREBP1 in cell culture:

For Western blotting, polyclonal antibodies against SCD and SREBP1 were prepared for which a 591 bp length of *SCD* cDNA and a 507 bp length of *SREBP1* cDNA were amplified by designing specific primers (SCD_Ab Forward: AAGCTTATGCACCACCATCACCATCATAATATCCTCATGAGCCTG; SCD_Ab Reverse: GGATCCAAACATGTGAGCGCTG; SREBP1_Ab Forward: AAGCTTATGCACCACCATCACCATCATCCTGACAGCACCCTGTC; SREBP1_Ab Reverse: GGATCCGCTCTGCCTTGATGAAGTG). The forward primers of both cDNAs contained with 6X histidine tag and *Bam*HI restriction enzyme while the reverse primers contained *Hind* III restriction enzyme. The PCR amplification was carried out in 200 µL PCR tubes containing 2.5 µL of 10X buffer, 1 µL of dNTP mix (2.5mM), 1.5 µL (30ng) each of forward and reverse primers, 0.3 µL (1.5U) of Taq DNA polymerase, 1 µL of genomic DNA and nuclease free water to make up the volume up to 25 µL. Thermal cycling conditions followed for both the genes were initial denaturation at 95 °C for 10 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds and extension at 72 °C for 1 minute 30 sec and a final extension of 72 °C for 10 minutes. The amplified product was gel eluted and purified by using QIA quick gel extraction kit (Qiagen).

The pAcGFP1-C1 expression vector and purified amplicons of cDNA of both the genes were digested with *Bam*HI and *Hind* III restriction enzymes. After RE digestion, cDNA of *SCD* gene and *SREBP1* gene were cloned separately into the pAcGFP1-C1 expression vector. The ligated products were transformed into DH5α *E. coli* competent cells. The positive clones were screened and identified by colony PCR, plasmid PCR and sequencing. The recombinant plasmids were isolated by using Gene JET plasmid miniprep kit (Thermo Scientific, USA). The recombinant plasmids of *SCD* and *SREBP1* genes were transfected into the CEH by using the Gene Pulser Xcell™ Electroporation system (Biorad). After transfection, hepatocytes were grown in growth medium for 48 hrs and then, harvested for isolation of proteins. From the cell lysates, both *SCD* and *SREBP1* proteins tagged with 6x histidine were extracted by using the His-Spin Protein Miniprep™ kit (GCC Biotech, Kolkata, India).

The purified protein was mixed with Freund's Adjuvant (IFA) and the mixture was injected subcutaneously (s/c). Primary injection was given with Freund's Complete Adjuvant (CFA) and then, booster injections were given with Incomplete Freund's Adjuvant (IFA). A total of 6 male Wistar rats (2 for *SCD* protein, 2 for *SREBP1* protein and 2 as control) of 8 weeks age were included in immunization schedule throughout period. The detailed Immunization protocol for rat polyclonal antibody production was as shown in Table S1. The IgG was purified from hyper immune sera using IgG purification kit (Himedia).

A period of 48 h after transfection of chicken embryonic hepatocyte cells with shRNAs against *SCD* and *SREBP1* genes, cell pellets were harvested. The cell pellets were washed in ice cold PBS solution and then cell lysate was prepared by adding cold cell lysis buffer (150 mMNaCl, 1% NP-40, and 50 mM Tris, pH

7.4) @ 1ml per 150 cm² flask, agitated constantly and centrifuged at a rate of 12,000 rpm, at 4 °C for 20 min. Equal volume of Laemmli 2X sample loading buffer (10% SDS, 0.025% Bromophenol blue and 1% DTT) added to the obtained cell lysate and boiled at 100 °C for 3 min. About 20 µl of digested samples containing approximately 15–20 µg of protein loaded into the wells of SDS-PAGE with discontinuous buffer system Tris–Glycine–SDS buffer, pH 8.3 to separate the protein mixture. After completion of electrophoresis, the gel containing protein was transferred on to the polyvinylidene fluoride (PVDF) membrane in the presence of Tris–Glycine–Methanol Buffer. After careful transfer of the gel, the blotted PVDF was immersed in 3% BSA blocking buffer with primary antibody (1:1000 dilution in TBST) and incubated at 4°C for overnight. Then the membrane was washed three times with the TBS Tween 20 (TBST) for 5 minutes for each wash. After washing, membrane was incubated with anti-rat IgG HRP conjugate (Sigma) diluted to 1:1000 in TBS Tween 20 buffer for 1.5 hours with constant agitation. After washings with the TBS Tween 20 (TBST), the PVDF membrane was incubated in DAB substrate solution for 5-30 minutes until the color development. Soon after the appearance of brown color, substrate solution was drained and the reaction was stopped by adding distilled water.

Sandwich ELISA for quantitation of SCD and SREBP1 in cell culture:

IgGs specific for *SCD* and *SREBP1* have been pre-coated onto a 96-well plate (12 x 8 Well Strips) and blocked separately. Test samples (cell lysate obtained after transfecting the shRNA molecules into the hepatocytes) were added to the wells, incubated and removed. HRP detector antibodies specific for *SCD* and *SREBP1* were added, incubated and followed by washing. HRP-Peroxidase Conjugate was then added, incubated and unbound conjugate was washed away. An enzymatic reaction was produced through the addition of TMB substrate which is catalyzed by HRP generating a blue color product that changes yellow after adding acidic stop solution. The density of yellow coloration read by absorbance at 450 nm is quantitatively proportional to the amount of sample *SCD* and *SREBP1* captured in well.

Statistical analysis:

The statistical analysis in the experiments was carried out using trial version of SPSS 20. Univariate General linear model with Tukey's HSD and DMRT as post-hoc test was used to study the significant difference between different shRNA groups due to the knock down effect of target genes. Data from representative experiments were presented as Mean ± SE for different samples with differences determined by least significant differences at 5% level ($P < 0.05$). The degree of association between the expression of different genes was calculated by Pearson correlation coefficient.

Results

shRNA molecules:

A total of 5shRNA molecules for each of *SCD* and *SREBP1* genes were designed and synthesized. Local secondary structures of target mRNA sequences of *SCD* and *SREBP1* genes were predicted and they revealed that all the shRNA target mRNAs consist of stem and loop secondary structures (Figure.1 and Figure.2). Analysis of secondary structures of shRNA molecules designed against *SCD* and *SREBP1* genes revealed different types of secondary structures (Figure.3 and Figure.4).

The predicted values (negative) of ΔG overall, ΔG duplex and ΔG break–target (disruption energy) for mRNA target regions of *SCD* and *SREBP1* genes were within the desirable range (Table 3 & Table 4). In case of *SCD* gene target mRNA region, overall ΔG value was almost same for shRNA2, shRNA4 and shRNA5. ΔG duplex value was the highest for shRNA5 and lowest for shRNA1. ΔG break–target (disruption energy) was highest for shRNA3, lowest for shRNA2. For *SREBP1* gene mRNA target regions, overall ΔG value was highest for shRNA1, lowest for shRNA2. ΔG duplex value was highest for shRNA4 and lowest for shRNA1. ΔG break–target (disruption energy) was highest for shRNA2 and lowest for shRNA1. GC content of the shRNA target mRNA regions of *SCD* gene ranged from 43% to 52%, whereas, GC content of the shRNA target mRNA regions of *SREBP1* gene ranged from 48% to 52% (Table 3 & Table 4).

A volume of 5 µl of 500 nM stock of ds oligos from each of the shRNA molecule was loaded on to the 4% agarose gel for checking the integrity of the annealed ds oligos (Figure.S3 & Figure.S4). Annealed ds oligos were observed at 50 bp length, unannealed single-stranded oligos were observed at 25 bp length as the agarose gel is non-denaturing. Therefore, the single-stranded oligos do not resolve at the expected size due to formation of secondary structure. Successfully annealed ds oligos were used further for cloning into the pENTR /U6 Entry Vector.

RNAi cassettes for production of shRNAs:

Successfully annealed ds oligos were used for cloning into RNAi-Ready pENTR/U6 entry vector. After successful ligation of annealed ds oligos into RNAi-Ready pENTR/U6 entry vector, recombinant clones were transformed into competent *E. coli* cells. Recombinant clones were confirmed by colony PCR (Figure.S5 & Figure.S6) and plasmid PCR (Figure.S7 & Figure.S8) on 1.5 % agarose gel and sequencing.

Transient transfection of anti-SCD and -SREBP1 shRNA construct into CEH:

The hepatocytes were transfected with anti-*SCD* and -*SREBP1* shRNA constructs separately and grown in DMEM medium. The cells were harvested and then, total RNA and DNA were isolated from the harvested hepatocytes. For confirmation of successful transfection of recombinant shRNA molecules, DNA extracted from the harvested hepatocytes was used as template for performing the PCR with specific primers for U6 Entry vector. The PCR products found at a product length of 287 bp confirmed the successful transfection of the recombinant shRNA constructs into the hepatocytes (Figure.S9 & Figure.S10). The total RNA isolated from the hepatocytes into which recombinant shRNA constructs were transfected, was used for the synthesis of first strand of cDNA. The cDNA was used for performing Real time PCR for assessing the expression of *SCD*, *SREBP1* and *GAPDH* genes in the hepatocytes after transfecting with recombinant shRNA constructs.

Silencing efficiency of anti-*SCD* and -*SREBP1* shRNA constructs

A significant ($P < 0.05$) reduction was observed at the mRNA expression levels of *SCD* and *SREBP1* genes, after transfecting the recombinant shRNA constructs into the hepatocytes, when compared with mock control. Expression levels ($40 - \Delta Ct$) of *SCD* gene after transfecting with different shRNA constructs was 37.97, 37.61, 38.76, 38.42, 39.235 and 39.565 in the cells with shRNA1, shRNA2, shRNA3, shRNA4, shRNA5 and mock control, respectively. Expression of *SCD* gene in the transfected hepatocytes with shRNA1 and shRNA2 molecules reduced significantly ($P < 0.05$) when compared with the mock control. Knock down efficiency of shRNA1, shRNA2, shRNA3, shRNA4 and shRNA5 molecules were 66.90%, 74.21%, 42.76%, 54.78% and 20.45%, respectively (Figure 9). The shRNA2 molecule was found as the best molecule among all the shRNA molecules used for silencing the *SCD* gene expression *in vitro*.

In case of *SREBP1* gene, mRNA expression levels ($40 - \Delta Ct$) were 29.58, 33.60, 32.31, 33.72, 33.64 and 34.165 after transfecting with shRNA1, shRNA2, shRNA3, shRNA4, shRNA5 molecules into embryonic hepatocytes and mock control respectively. Expression levels of *SREBP1* gene in hepatocytes into which shRNA1, shRNA2, shRNA3 shRNA4 and shRNA5 molecules were transfected, reduced significantly ($P < 0.05$) when compared with mock control. Knock down efficiency of shRNA1, shRNA2, shRNA3, shRNA4 and shRNA5 molecules were 95.85%, 32.64%, 72.36%, 26.80% and 30.50% respectively (Figure 10). The shRNA1 molecule was found as the best among all the shRNA molecules used for silencing the *SREBP1* gene under cell culture system. However, Western blot analysis also revealed the expression of *SCD* and *SREBP1* proteins in both transfected as well as non-transfected mock control (Figure 5 and Figure 6).

Relative quantification of immune response genes in transfected hepatic cells

The relative expression of immune response genes (*IFNA* and *IFNB*) was monitored by quantitative PCR, after transfecting the anti-*SCD* and anti-*SREBP1* shRNA constructs into the hepatocytes. Gene expression levels of *IFNA* ($40 - \Delta Ct$) was 38.525, 37.93, 37.355, 36.57, 41.23 and 34.015, while that of *IFNB* was 36.385, 35.61, 34.755, 34.815, 39.64 and 33.04 in anti-*SCD* shRNA constructs viz. shRNA1, shRNA2, shRNA3, shRNA4, shRNA5 and Scrambled shRNA, respectively (Figure 7). In case of anti-*SREBP1* shRNA constructs, the mRNA levels of *IFNA* were 35.44, 39.075, 40.2, 39.615, 40.115 and 38.23 while that of *IFNB* was 34.28, 37.5, 38.55, 38.185, 38.42 and 37.35 in the hepatocyte cells transfected with shRNA1, shRNA2, shRNA3, shRNA4, shRNA5 and Scrambled shRNA, respectively (Figure 8). There was no significant ($P < 0.05$) difference noticed in the expression of immune response genes when compared between shRNA transfected cells with the scrambled shRNA.

Detection of *SCD* and *SREBP1* protein in cell culture:

The sandwich ELISA results revealed that, the colour reaction was observed at a titre of 1:250, 1:100, 1:500, 1:500, 1:750 and 1:1000 in transfected cells with anti-*SCD* shRNA1, shRNA2, shRNA3, shRNA4, shRNA5 and scrambled shRNA (Figure 9). In case of anti-*SREBP1* shRNA1, shRNA2, shRNA3, shRNA4, shRNA5 and scrambled shRNA, the titre was 1:10, 1:500, 1:100, 1:500, 1:500 and 1:1000, respectively (Figure 10). As shRNA molecules efficiently reduced the production of *SCD* and *SREBP1* protein synthesis, those molecules which have the higher knock down efficiency (shRNA2 for *SCD*; shRNA1 and for *SREBP1*) exhibited colour reaction at lower dilutions of protein.

Discussion

RNA interference (RNAi) has become one of the powerful tools in recent times to suppress the expression of specific gene of interest [17–18], as a therapeutic option in disease management [19–22], as cancer treatment [23–25] and for understanding regulatory function of a gene by dsRNA molecules [26]. Suppression of gene expression achieved by cleavage of dsRNA molecules by ribonuclease protein (Dicer) followed by loading of siRNA molecules into RISC (RNA induced silencing complex) and then, guide strand of the siRNA molecule would guide the RISC towards the target mRNA sequence complementary with the siRNA for cleavage of target mRNA [27–31]. For successful reduction of gene expression through RNAi, designing of shRNA molecules against target gene plays crucial role [32]. In our study, anti shRNA molecules against *SCD* and *SREBP1* genes were synthesized following the criteria such as moderate to low GC content, low internal stability of sense strand at 3' end (at least 3 bases of A/U at 15–19 positions of sense strand) and lack of internal repeats [33]. Basic Local Alignment Search Tool (BLAST) was used to detect whether our designed shRNA molecules are having the homology with any other genes to avoid the potential off-target effects.

Earlier studies revealed that the formation of secondary structures in the anti-sense/guide strand of shRNA molecule had critical role in determining the silencing efficiency and stated that a strong inverse correlation was observed between the degree of formation guide-RNA secondary structure formation and gene silencing efficiency of shRNA [34]. Patzel *et al.* 2005 classified and reported the secondary structures of guide strands of shRNA as: best silencing exhibited by sequences without any secondary structures, second best were stem loop structures with ≥ 2 free nucleotides at 5' and ≥ 4 free nucleotides at 3' end, next best were sequences having internal loop and two stem loop structures followed by stem loop structures with short free ends [34]. In accordance with the previous findings in our study, the shRNA molecules with best silencing efficiency (i.e shRNA2 molecule for *SCD* gene and shRNA1 molecule for *SREBP1* gene) did not possess any secondary structure in their anti-sense/guide strands. Second best shRNA molecules (i.e shRNA1 molecule for *SCD* gene and shRNA3 molecule for *SREBP1* gene) have loop structures with ≥ 2 free nucleotides at 5' and ≥ 4 free nucleotides at 3' end. Some of the previous studies in chicken also reported that shRNA molecules having no secondary structures in their anti-sense/guide strand had expressed high knock down efficiency in other genes such as myostatin gene and ActRIIB gene [35–36, 32].

Earlier studies suggested that the local structure of mRNA is one of the key factors and had a strong effect on the silencing efficiency of the shRNA molecule [37–42]. All the mRNA target regions of anti-*SCD* and anti-*SREBP1* shRNA molecules revealed stem loop structures. Some studies inferred that presence of loop structures in the target mRNA regions provide easy access to the guide strand to bind with target region and had positive correlation with gene silencing efficiency [41, 43]. Presence of paired nucleotides and hairpins in the secondary structure of target mRNA region likely to have negative effect on shRNA silencing [39, 41]. In our study even though all target mRNA regions of *SCD* and *SREBP1* genes have accessibility, the differences in the silencing efficiency might be because of other responsible factor.

GC content of the target mRNA region have the crucial role in determining the silencing efficiency and it influences the loading of shRNA molecule into RISC and target affinity and specificity [16]. A high GC content in shRNA molecule hinders the dissociation of siRNA duplex inhibiting RISC loading [44–45]. In contrary to that, some reports stated that a low GC content may reduce silencing efficiency by low target affinity and specificity [46]. The GC content of target mRNA region of our target genes ranged from 43–52%. Previous studies also suggested that GC content between 30% and 55% had positive correlation with silencing [33, 47, 48].

The shRNA molecules possessing high knock down efficiency must have higher negative value of ΔG overall, ΔG duplex and lesser negative value of ΔG break-target (disruption energy) [18, 32]. Some studies postulated that shRNA molecules having the values of ΔG overall, ΔG duplex and ΔG break-target (disruption energy) within the range of – 25 to – 35 kcal/mol, – 30 to – 40 kcal/mol and – 0.5 to – 1.5 kcal/mol exhibited good silencing efficiency [32, 49]. In our study, all the shRNA molecules designed against *SCD* and *SREBP1* mRNA possessing the values of ΔG overall, ΔG duplex and ΔG break-target (disruption energy) within the desirable range have shown good silencing efficiency. The shRNA2 molecule found as the best in silencing the expression of *SCD* mRNA having higher negative value of ΔG overall, ΔG duplex and lesser negative value of ΔG break-target (disruption energy) among all shRNAs designed. Similar findings were observed in case of shRNA1 molecule, which was found as the best one in silencing the expression of *SREBP1* gene expression. Possessing the thermodynamic properties within the desirable range might be the one of the reasons for better silencing efficiency of shRNA2 in *SCD* and shRNA1 in *SREBP1* genes.

Silencing of the genes which are involved in the fatty acid biosynthesis has benefits like production of lean meat and egg and reduction of fat deposition in several organs of the birds. Silencing of target genes which are involved in the fat biosynthesis by using RNA interference in chicken primary hepatocyte culture helped in the devising suitable *in vitro* models for knock down of the target gene. This technique can be used further for production of the knock down chicken with reduced fat production. Till date, there were no reports available in the literature regarding the silencing of fatty acid synthesis genes in chicken. In the present study, the shRNA molecules were successfully transfected into the chicken hepatic cells and significant down regulation of the target genes were observed. These results suggest that the chicken embryo hepatocytes can be used for *in vitro* model for various functional studies. Significant reduction in the expression of *SCD* and *SREBP1* genes in hepatocytes was observed after transfecting the shRNA molecules into them. The shRNA constructs against *SCD* gene showed the knock down efficiency ranged from 20.4–74.2%. In case of shRNA constructs against *SREBP1* gene, they showed knock down efficiency ranging from 26.8–95.8%. The reasons behind the high knock down efficiency of shRNA2 against *SCD* gene and shRNA1 molecule against *SREBP1* gene might be lack of secondary structures in their anti-sense/guide strand, desirable GC percentage (43%–52%) in sense strand, more accessibility in the target mRNA region i.e stem loop structures in secondary structure of mRNA region and possessing thermodynamic properties falling in desirable range. The expression of the *SCD* and *SREBP1* examined in the cell lysates showed lower expression in the cells transfected with shRNAs indicating the similar trends as of mRNA expression in the cell culture. Thus, these results clearly demonstrated the successful down regulation of the gene expression by designed shRNA molecules against both the target genes under *in vitro* condition. In previous reports, by using shRNA molecules in Myostatin gene [50], *ACTRIIA* gene [51] and *ACTRIIB* gene [32] knock down efficiency of 68%, 87% and 82% were found respectively in chicken embryo fibroblasts cells. In duck embryo fibroblasts, different shRNA molecules mediated through lenti-virus have down regulated the *MSTN* gene expression by 61.6, 76.9 and 79.1%, respectively, when compared with control cells [52]. In caprine foetal fibroblasts, mRNA expression levels of myostatin gene were down regulated by 89% [53] and 72% [54] after transient transfection of shRNA molecules into them.

RNA interference is powerful technique for down regulating the particular gene but it may cause the activation of immune response genes under *in vitro* and *in vivo* condition. According to the recent reports, both immune cells and non-immune cells can detect the shRNA molecules irrespective of their sequence and leads to the stimulation of interferon and inflammatory cytokines under *in vivo* and *in vitro* conditions [55, 56]. It was also reported that, shRNA molecules shorter than size of 30 bp can escape the PKR activation. It is known that the IFN response caused by activation of protein kinase R (PKR), ultimately leads to the inhibition of protein synthesis [56]. In the present study, we observed the expression of immune response genes particularly *IFNA* and *IFNB* in both tranfected and non-transfected control cells in case of both *SCD* and *SREBP1* experiments. In both the genes, the non-significant differences of expression of *IFNA* and *IFNB* genes between shRNA transfectd cells and either scrambled shRNA transfected cells or un-transfected negative control embryonic hepatocyte cells. In supporting to the results obtained in the present study, Patel *et al.* (2014) and Guru Vishnu *et al.* (2019) in *ACTIIB* gene in goat and chicken fibroblast cells, respectively [57, 32]. We suggest that shRNAs designed in our study against both *SCD* and *SREBP1* genes had the potential to be excellent shRNA molecules for further development of knock down chicken as the shRNA molecules having very good knock down efficiency escaped the interferon system of the cells.

Conclusion

It is concluded that short hairpin RNA-based silencing of *SCD* and *SREBP1* genes under *in vitro* chicken hepatic cell culture system have been developed to minimize expression of these genes. The knock-down efficiency for both the genes reached more than 70% under *in vitro* cell culture system without affecting expression of immune response genes.

Declarations

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Ethical Approval

The whole study was approved by the Institute Animal Ethics Committee (IAEC) to carry out the animal experiment.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Authors Contributions

NGS has done wet lab experiment. ARP has done cell culture work. PK has analysed the data. BB has prepared all the tables. PGV has also helped in data analysis. TKB conceived the idea, planned the experiments and prepared the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

Availability of data and materials

The data and materials that support the findings of this study are available as the supplementary material of this article and in the lab.

References

1. Havenstein, G. B., Ferket, P. R., & Qureshi, M. A. (2003). Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poultry science*, 82, 1500–1508
2. Zerehdaran, S. A. L. J., Vereijken, A. J., Van Arendonk, J. A. M., & Van der Waaijt, E. H. (2004). Estimation of genetic parameters for fat deposition and carcass traits in broilers. *Poultry science*, 83, 521–525
3. Siegel, P. B., & Wolford, J. H. (2003). A review of some results of selection for juvenile body weight in chickens. *The Journal of Poultry Science*, 40, 81–91
4. Hermier, D. (1997). Lipoprotein metabolism and fattening in poultry. *The Journal of nutrition*, 127, 805–808
5. Whitehead, C. C., & Griffin, H. D. (1984). Development of divergent lines of lean and fat broilers using plasma very low-density lipoprotein concentration as selection criterion: the first three generations. *British Poultry Science*, 25, 573–582
6. Griffin, H. D., Guo, K., Windsor, D., & Butterwith, S. C. (1992). Adipose tissue lipogenesis and fat deposition in leaner broiler chickens. *The Journal of nutrition*, 122, 363–368
7. Enoch, H. G., Catala, A., & Strittmatter, P. (1976). Mechanism of rat liver microsomal stearyl-CoA desaturase. Studies of the substrate specificity, enzyme-substrate interactions, and the function of lipid. *Journal of Biological Chemistry*, 251, 5095–5103
8. Peláez, R., Pariente, A., Pérez-Sala, Á., & Larráyoz, I. M. (2020). Sterculic acid: the mechanisms of action beyond stearyl-CoA desaturase inhibition and therapeutic opportunities in human diseases. *Cells*, 9, 140
9. Seppälä-Lindroos, A., Vehkavaara, S., Häkkinen, A. M., Goto, T., Westerbacka, J., Sovijärvi, A. ... Yki-Järvinen, H. (2002). Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *The Journal of Clinical Endocrinology & Metabolism*, 87, 3023–3028
10. Brown, W. R., Hubbard, S. J., Tickle, C., & Wilson, S. A. (2003). The chicken as a model for large-scale analysis of vertebrate gene function. *Nature Reviews Genetics*, 4, 87–98
11. Bertolio, R., Napoletano, F., Mano, M., Maurer-Stroh, S., Fantuz, M., Zannini, A. ... Del Sal, G. (2019). Sterol regulatory element binding protein 1 couples mechanical cues and lipid metabolism. *Nature communications*, 10, 1–11
12. Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic acids research*, 31, 3406–3415
13. Lorenz, R., Bernhart, S. H., Siederdisen, Z., Tafer, C. H., Flamm, H., Stadler, C., & Hofacker, I. L., P. F., &. (2011). ViennaRNA Package 2.0. *Algorithms for molecular biology*, 6, 1–14
14. Mathews, D. H., Burkard, M. E., Freier, S. M., Wyatt, J. R., & Turner, D. H. (1999). Predicting oligonucleotide affinity to nucleic acid targets. *Rna*, 5, 1458–1469
15. Reuter, J. S., & Mathews, D. H. (2010). RNAstructure: software for RNA secondary structure prediction and analysis. *BMC bioinformatics*, 11, 1–9
16. Pascut, D., Bedogni, G., & Tiribelli, C. (2015). Silencing efficacy prediction: a retrospective study on target mRNA features. *Bioscience reports*, 35.
17. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *nature*, 391, 806–811
18. Hannon, G. J. (2002). *RNA interference*. *nature*, 418, 244–251
19. Acharya, R. (2019). The recent progresses in shRNA-nanoparticle conjugate as a therapeutic approach. *Materials Science and Engineering: C*, 104, 109928

20. Acharya, R. (2020). Prospective vaccination of COVID-19 using shRNA-plasmid-LDH nanoconjugate. *Medical Hypotheses*, 143, 110084
21. Kumar, S., Dey, A., Yau, Y. Y., Easterling, M., & Sahoo, L. (2020). RNA Interference: For Improving Traits and Disease Management in Plants. In *Climate Change, Photosynthesis and Advanced Biofuels* (pp. 339–368). Singapore: Springer
22. Thijssen, M. F., Brüggerwirth, I. M., Gillooly, A., Khvorova, A., Kowalik, T. F., & Martins, P. N. (2019). Gene silencing with siRNA (RNA interference): a new therapeutic option during ex vivo machine liver perfusion preservation. *Liver Transplantation*, 25, 140–151
23. Ni, Q., Zhang, F., Zhang, Y., Zhu, G., Wang, Z., Teng, Z. ... Chen, X. (2018). In situ shRNA synthesis on DNA–polylactide nanoparticles to treat multidrug resistant breast cancer. *Advanced Materials*, 30, 1705737
24. Qiao, H., Wang, Y. F., Yuan, W. Z., Zhu, B. D., Jiang, L., & Guan, Q. L. (2018). Silencing of ENO1 by shRNA inhibits the proliferation of gastric cancer cells. *Technology in cancer research & treatment*, 17, 1533033818784411
25. Shailender, G., Patanla, K., & Malla, R. R. (2020). ShRNA-mediated matrix metalloproteinase-2 gene silencing protects normal cells and sensitizes cancer cells against ionizing-radiation induced damage. *Journal of cellular biochemistry*, 121, 1332–1352
26. Patel, M., & Peter, M. E. (2018). Identification of DISE-inducing shRNAs by monitoring cellular responses. *Cell Cycle*, 17, 506–514
27. Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, 409, 363–366
28. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *nature*, 411, 494–498
29. Hammond, S. M., Bernstein, E., Beach, D., & Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature*, 404, 293–296
30. Hammond, S. M., Caudy, A. A., & Hannon, G. J. (2001). Post-transcriptional gene silencing by double-stranded RNA. *Nature Reviews Genetics*, 2, 110–119
31. Zamore, P. D., Tuschl, T., Sharp, P. A., & Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, 101, 25–33
32. Vishnu, P. G., Bhattacharya, T. K., Bhushan, B., Kumar, P., Chatterjee, R. N., Paswan, C. ... Prasad, A. R. (2019). In silico prediction of short hairpin RNA and in vitro silencing of activin receptor type IIB in chicken embryo fibroblasts by RNA interference. *Molecular biology reports*, 46, 2947–2959
33. Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., & Khvorova, A. (2004). Rational siRNA design for RNA interference. *Nature biotechnology*, 22, 326–330
34. Patzel, V., Rutz, S., Dietrich, I., Köberle, C., Scheffold, A., & Kaufmann, S. H. (2005). Design of siRNAs producing unstructured guide-RNAs results in improved RNA interference efficiency. *Nature biotechnology*, 23, 1440–1444
35. Bhattacharya, T. K., Shukla, R., Chatterjee, R. N., & Dushyanth, K. (2017). Knock down of the myostatin gene by RNA interference increased body weight in chicken. *Journal of biotechnology*, 241, 61–68
36. Tripathi, A. K., Aparnathi, M. K., Patel, A. K., & Joshi, C. G. (2013). In vitro silencing of myostatin gene by shRNAs in chicken embryonic myoblast cells. *Biotechnology progress*, 29, 425–431
37. Bohula, E. A., Salisbury, A. J., Sohail, M., Playford, M. P., Riedemann, J., Southern, E. M., & Macaulay, V. M. (2003). The efficacy of small interfering RNAs targeted to the type 1 insulin-like growth factor receptor (IGF1R) is influenced by secondary structure in the IGF1R transcript. *Journal of Biological chemistry*, 278, 15991–15997
38. Gredell, J. A., Berger, A. K., & Walton, S. P. (2008). Impact of target mRNA structure on siRNA silencing efficiency: a large-scale study. *Biotechnology and bioengineering*, 100, 744–755
39. Kretschmer-Kazemi Far, R., & Sczakiel, G. (2003). The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides. *Nucleic acids research*, 31, 4417–4424
40. Luo, K. Q., & Chang, D. C. (2004). The gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. *Biochemical and biophysical research communications*, 318, 303–310
41. Schubert, S., Grünweller, A., Erdmann, V. A., & Kurreck, J. (2005). Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions. *Journal of molecular biology*, 348, 883–893
42. Stewart, C. K., Li, J., & Golovan, S. P. (2008). Adverse effects induced by short hairpin RNA expression in porcine fetal fibroblasts. *Biochemical and biophysical research communications*, 370, 113–117
43. Overhoff, M., Alken, M., Far, R. K. K., Lemaitre, M., Lebleu, B., Sczakiel, G., & Robbins, I. (2005). Local RNA target structure influences siRNA efficacy: a systematic global analysis. *Journal of molecular biology*, 348, 871–881
44. Holen, T. (2005). Mechanisms of RNAi: mRNA cleavage fragments may indicate stalled RISC. *Journal of RNAi and gene silencing: an international journal of RNA and gene targeting research*, 1, 21
45. Matveeva, O., Nechipurenko, Y., Rossi, L., Moore, B., Sætrum, P., Ogurtsov, A. Y. ... Shabalina, S. A. (2007). Comparison of approaches for rational siRNA design leading to a new efficient and transparent method. *Nucleic acids research*, 35, e63
46. Li, W., & Cha, L. (2007). Predicting siRNA efficiency. *Cellular and molecular life sciences: CMLS*, 64, 1785–1792
47. Shah, J. K., Garner, H. R., White, M. A., Shames, D. S., & Minna, J. D. (2007). siR: siRNA Information Resource, a web-based tool for siRNA sequence design and analysis and an open access siRNA database. *BMC bioinformatics*, 8, 1–7
48. Wang, L., & Mu, F. Y. (2004). A Web-based design center for vector-based siRNA and siRNA cassette. *Bioinformatics*, 20, 1818–1820

49. Shao, Y., Chan, C. Y., Maliyekkel, A., Lawrence, C. E., Roninson, I. B., & Ding, Y. (2007). Effect of target secondary structure on RNAi efficiency. *Rna*, 13, 1631–1640
50. Tripathi, A. K., Aparnathi, M. K., Vyavahare, S. S., Ramani, U. V., Rank, D. N., & Joshi, C. G. (2012). Myostatin gene silencing by RNA interference in chicken embryo fibroblast cells. *Journal of biotechnology*, 160, 140–145
51. Satheesh, P., Bhattacharya, T. K., Kumar, P., Chatterjee, R. N., Dhara, S. K., Paswan, C. ... Dushyanth, K. (2016). Gene expression and silencing of activin receptor type 2A (ACVR2A) in myoblast cells of chicken. *British poultry science*, 57, 763–770
52. Tao, Z., Zhu, C., Song, C., Song, W., Ji, G., Shan, Y. ... Li, H. (2015). Lentivirus-mediated RNA interference of myostatin gene affects MyoD and Myf5 gene expression in duck embryonic myoblasts. *British poultry science*, 56, 551–558
53. Kumar, R., Singh, S. P., Kumari, P., & Mitra, A. (2014). Small interfering RNA (siRNA)-mediated knockdown of myostatin influences the expression of myogenic regulatory factors in caprine foetal myoblasts. *Applied biochemistry and biotechnology*, 172, 1714–1724
54. Jain, S. K., Jain, H., Kumar, D., Bedekar, M. K., Pandey, A. K., & Sarkhel, B. C. (2015). Quantitative evaluation of myostatin gene in stably transfected caprine fibroblast cells by anti-myostatin shRNA. *Applied biochemistry and biotechnology*, 177, 486–497
55. Sledz, C. A., Holko, M., De Veer, M. J., Silverman, R. H., & Williams, B. R. (2003). Activation of the interferon system by short-interfering RNAs. *Nature cell biology*, 5, 834–839
56. Judge, A. D., Sood, V., Shaw, J. R., Fang, D., McClintock, K., & MacLachlan, I. (2005). Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nature biotechnology*, 23, 457–462
57. Patel, A. K., Tripathi, A. K., Shah, R. K., Patel, U. A., & Joshi, C. G. (2014). Assessment of goat activin receptor type IIB knockdown by short hairpin RNAs in vitro. *Journal of Receptors and Signal Transduction*, 34, 506–512

Tables

Table 1. List of designed shRNA molecules against *SCD* and *SREBP1* genes. The shRNA molecules were designed using publicly available sequences at NCBI Accession No. NC_006093 for *SCD* and NCBI Accession No. NC_006101 for *SREBP1* genes.

shRNA ID	Nucleotide sequence (5' to 3')
<i>SCD</i>	
Lac Z_top	CACCGCTACACAAATCAGCGATTT CGA Aaaatcgctgattgtgtag
Lac Z_bottom	AAAAC TACACAAATCAGCGATTTTT CGAAATCGCTGATTGTGTAGC
140_top	CACCGCATGGTAGATGATCTCTTTG CGA Acaaagagatcatctaccatgc
140_bottom	AAAAGCATGGTAGATGATCTCTTTGTT CGCAAAGAGATCATCTACCATGC
201_top	CACCGGATACGTCTGGAGGAATAT CGA Aatattcctccagacgtatcgc
201_bottom	AAAAGCGATACGTCTGGAGGAATATTT CGATATTCCTCCAGACGTATCGC
362_top	CACCGGAGCCATCGATCCTACAAAG CGA Actttgtaggatcgatggctcc
362_bottom	AAAAGGAGCCATCGATCCTACAAAGTT CGCTTTGTAGGATCGATGGCTCC
496_top	CACCGCAGACCCTCACAATGCTATG CGA Acatagcattgtgagggctctgc
496_bottom	AAAAGCAGACCCTCACAATGCTATGTT CGCATAGCATTGTGAGGGTCTGC
591_top	CACCGCTGGACCTGAGTGATTTAAA CGA Atttaaatcactcaggtccagc
591_bottom	AAAAGCTGGACCTGAGTGATTTAAATTCGTTTAAATCACTCAGGTCCAGC
<i>SREBP1</i>	
114_top	CACCGATCAACACACCGGACAATGAC GA Attcattgtccggtgtgttgatc
114_bottom	AAAAGATCAACACACCGGACAATGATTCGTCATTGTCCGGTGTGTTGATC
935_top	CACCGCTCATCCATCAACGACAAGA CGA Attctgtcggtgatgatgagc
935_bottom	AAAAGCTCATCCATCAACGACAAGATT CGTCTTGTCGTTGATGGATGAGC
1050_top	CACCGCAGAGCAACCAGAAGCTGAA CGA Attcagcttctggttgctctgc
1050_bottom	AAAAGCAGAGCAACCAGAAGCTGAATTCGTT CAGCTTCTGGTTGCTCTGC
1074_top	CACCGGAGAACCTCAGCCTGAAGAT CGA Aatcttcaggctgaggttctcc
1074_bottom	AAAAGGAGAACCTCAGCCTGAAGATTT CGATCTTCAGGCTGAGGTTCTCC
2430_top	CACCGGATGCGTTGGAGTACCTTCA CGA Attgaaggactccaacgcatcc
2430_bottom	AAAAGGATGCGTTGGAGTACCTTCAATTCGTTGAAGGTACTCCAACGCATCC

Bold upper case letters – sense target sequence, Lower case letter – anti sense sequence, Italic letters – Loop

Table 2. List of primers used for real time expression quantification of SCD, SREBP1 and immune response genes

Gene Symbol	Primer Sequences (5'– 3')	Amplicon Size (bp)	Annealing Temperature (°C)	Accession Number
<i>SCD</i>	F: TGACGCTGATCCCTTCTGC R: AATAGTCAAGAAGATCCGCAG	152	57	NC_006093
<i>SREBP1</i>	F: ACGACATGCTGCAGCTGATC R: GGGAAACACATTGCCGGTAG	160	59	NC_006101
<i>GAPDH</i>	F: CTGCCGTCCTCTCTGGC R: GACAGTGCCCTTGAAGTG	119	58	NM_204305
<i>IFNA</i>	F: CTGCTCACGCTCCTTCTG R: GTGTCTGTTGAAGGAGCAAG	170	58	NM_205427
<i>IFNB</i>	F: CTCCTTCAGAATACGGCTC R: GTGTGTGGGCTGCTAAGC	164	58	NM_001024836

Table 3. Thermo Dynamic properties & knock down efficiencies of anti-SCD shRNA molecules in chicken embryonic hepatic cell culture.

S.No	Molecule	Target mRNA sequence	Position on mRNA	Secondary structure of target mRNA	Target Exon	GC Content(%)	Thermodynamic Parameters		
							Overall ΔG	Duplex ΔG	E _t ΔG
1	shRNA1	GCATGGTAGATGATCTCTTTG	140-160	Stem+Loop	2	43	-28	-32	-32
2	shRNA2	GCGATACGTCTGGAGGAATAT	201-221	Stem+Loop	2	48	-30.7	-32.8	-32.8
3	shRNA3	GGAGCCATCGATCCTACAAAG	362-382	Stem+Loop	3	52	-28.2	-33.3	-33.3
4	shRNA4	GCAGACCCTCACAATGCTATG	496-516	Stem+ Large Loop	4	52	-30.6	-33.7	-33.7
5	shRNA5	GCTGGACCTGAGTGATTAAA	591-611	Stem+ 2 Loops	5	43	-30.8	-33.8	-33.8

Table 4. Thermo Dynamic properties & knock down efficiencies of anti-SREBP1 shRNA molecules in chicken embryonic hepatic cell culture.

S.No	Molecule	Target mRNA sequence	Position on mRNA	Secondary structure of target mRNA	Target Exon	GC Content(%)	Thermodynamic Parameters		
							Overall ΔG	Duplex ΔG	E _t ΔG
1	shRNA1	GATCAACACACCGGACAATGA	114-134	Stem+Loop	2	48	-30	-32.8	-32.8
2	shRNA2	GCTCATCCATCAACGACAAGA	935-955	Stem+ Loops	6	48	-24.1	-33	-33
3	shRNA3	GCAGAGCAACCAGAAGCTGAA	1050-1070	Stem+Loop	7	52	-27.4	-33.9	-33.9
4	shRNA4	GGAGAACCTCAGCCTGAAGAT	1074-1094	Stem+Loop	7	52	-25.9	-34.3	-34.3
5	shRNA5	GGATGCGTTGGAGTACCTTCA	2430-2450	Stem+Loop	15	52	-24.2	-33.6	-33.6

Figures

Figure 1

Local secondary structures of *SCD* mRNA targeted by shRNA1 – A, shRNA2 – B, shRNA3 – C, shRNA4 – D and shRNA5 – E. Bold and under lined regions – mRNA region targeted by shRNA.

Figure 2

Local secondary structures of *SREBP1* mRNA targeted by shRNA1 – A, shRNA2 – B, shRNA3 & shRNA4 – C and shRNA5 – D. Bold and under lined regions – mRNA region targeted by shRNA.

Figure 3

Secondary structures of anti sense/guide strand of different shRNA molecules designed against *SCD* mRNA.

Figure 4

Secondary structures of anti sense/guide strand of different shRNA molecules designed against *SREBP1* mRNA.

Figure 5

Western blot analysis of *SCD* expression in transfected chicken embryo hepatocytes. Lane sh1: shRNA1 transfected cells, Lane sh2: shRNA2 transfected cells, Lane sh3: shRNA3 transfected cells, Lane sh4: shRNA4 transfected cells, Lane sh5: shRNA5 transfected cells. Arrow indicates presence of blot of SCD protein in cell culture.

Figure 6

Western blot analysis of *SREBP1* expression in transfected chicken embryo hepatocytes. Lane sh1: shRNA1 transfected cells, Lane sh2: shRNA2 transfected cells, Lane sh3: shRNA3 transfected cells, Lane sh4: shRNA4 transfected cells, Lane sh5: shRNA5 transfected cells. Arrow indicates presence of blot of *SREBP1* protein in cell culture.

Figure 7

Expression of immune response genes (IFNA and IFNB) in hepatocytes transfected with anti-*SCD* shRNA constructs.

Figure 8

Expression of immune response genes (IFN A and IFN B) in hepatocytes transfected with anti-*SREBP1* shRNA constructs.

Figure 9

Knock down efficiencies of shRNA molecules of SCD gene and their associated ELISA titres in transfected and control chicken embryo hepatic cells.

Figure 10

Knock down efficiencies of shRNA molecules of SREBP1 gene and their associated ELISA titres in transfected and control chicken embryo hepatic cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigures.ppt](#)
- [SupplementaryTables.doc](#)
- [Supplementarydata1.xls](#)
- [Supplementarydata2.xls](#)